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Liquid Chromatographic Determination of Ciprofloxacin and Some Metabolites in Human Body Fluids

By *K. Borner*

Institut für Klinische Chemie und Klinische Biochemie

H. Lode, G. Höffken, C. Prinzing, P. Glatzel and *R. Wiley*

Medizinische Klinik und Poliklinik Klinikum Steglitz der Freien Universität Berlin

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Summary: Two column liquid chromatographic (HPLC) methods for the determination of ciprofloxacin and three metabolites are described. Both use reversed phase chromatography, the stationary phase being Nucleosil 5C18. Method A separates ciprofloxacin, metabolite M1 and another metabolite of unknown structure using fluorometric detection. Method B allows the determinations of metabolite M3 (oxo-ciprofloxacin) in urine by UV absorption. Serum was deproteinised with acetonitrile. Urine was diluted with buffer solution. The detection limit of ciprofloxacin was 0.010 mg/l serum and 0.2 mg/l urine and for the metabolite M3, 1 mg/l urine. Within-batch precision (coefficient of variation) for ciprofloxacin in serum was 0.8 to 2.4% and between-batch precision 4.8 to 9.3%. In urine within-batch precision was 1.7 to 2.1% and between-batch precision 2.4 to 7.2%. Recovery rates of ciprofloxacin from three groups of spiked sera was 94.5 ± 2.6%, 97.2 ± 1.1% and 95.0 ± 1.8% and from urine 99.6%. Results obtained by HPLC (method A) were compared with those from a standard microbiological assay by means of bivariate regression analysis. In 12 subsets of data the slope of the regression line varied from 1.042 to 1.556. Significantly higher results from the microbiological assay were probably due to the presence of microbiologically active metabolites. We conclude that HPLC is the more specific method of determination. The described methods were applied for pharmacokinetic studies and therapeutic drug monitoring.

Flüssigkeitschromatographische Bestimmung von Ciprofloxacin in menschlichen Körperflüssigkeiten

Zusammenfassung: Es werden zwei säulenchromatographische Methoden (HPLC) zur Bestimmung von Ciprofloxacin und von drei Metaboliten beschrieben. Beide Methoden arbeiten nach dem Prinzip der reversed phase Chromatographie. Die stationäre Phase ist Nucleosil 5C18. Methode A trennt Ciprofloxacin, Metabolit M1 und einen weiteren Metaboliten von bisher unbekannter Struktur und verwendet die Fluoreszenz zur Detektion. Methode B erlaubt die Bestimmung von Metabolit M3 (Oxo-ciprofloxacin) im Urin durch UV-Absorption. Serum wurde mit Acetonitril enteiweißt. Urin wurde mit Pufferlösung verdünnt. Die Nachweisgrenze von Ciprofloxacin war 0,01 mg/l Serum und 0,2 mg/l Urin. Die Nachweisgrenze von Metabolit M3 im Urin betrug 1 mg/l. Die Präzision in Serie, ausgedrückt als Variationskoeffizient, betrug für Ciprofloxacin im Serum 0,8 bis 2,4% und die Präzision von Tag zu Tag 4,8 bis 9,3%. Im Urin war die Präzision in Serie 1,7 bis 2,1% und die Präzision von Tag zu Tag 2,4 bis 7,2%. Die Wiederauffindung von Ciprofloxacin aus Serum war in drei Serien 94,5 ± 2,6%, 97,2 ± 1,1% und 95,0 ± 1,8%. Die Wiederauffindung aus Urin war 99,8%. Ergebnisse der HPLC-Methode A wurden mit den Ergebnissen eines üblichen mikrobiologischen Assays mit der bivariaten Regressionsanalyse verglichen. In 12 Daten-Gruppen variierte die Steigung der Regressionsgeraden von 1,042 bis 1,556. Die signifikant höheren Ergebnisse des Bioassays wurden vermutlich durch das Vorliegen von mikrobiologisch aktiven Metaboliten verursacht. Die HPLC ist unseres Erachtens die spezifischere Analysenmethode. Die beschriebenen HPLC-Methoden wurden zur Auswertung pharmakokinetischer Studien und im therapeutic drug monitoring eingesetzt.

Introduction

Ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl-)-quinolone-3-carboxylic acid; fig. 1) belongs to the recent derivatives of nalidixic acid, called gyrase inhibitors. It has remarkable antimicrobial activity (1—8) and is presently under clinical evaluation. This paper describes the determination of ciprofloxacin and three of its metabolites in human body fluids. The method has been extensively used in pharmacokinetic studies (9—11) and for therapeutic drug monitoring.

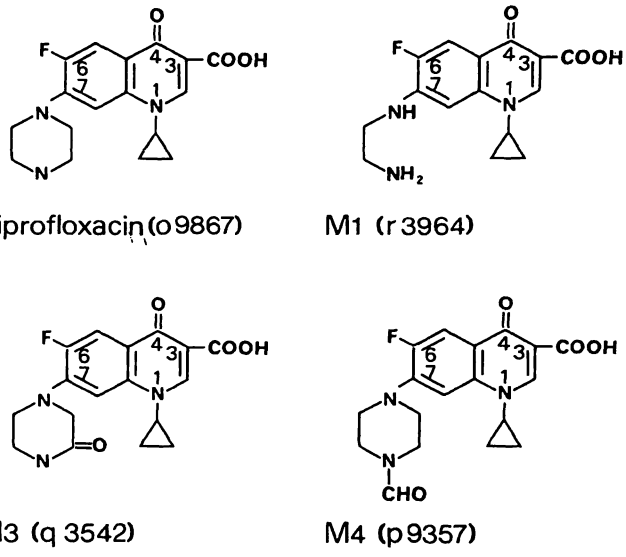

Fig. 1. Chemical structure of ciprofloxacin and some metabolites.

Methods

Chemicals

Reference material of ciprofloxacin (lot no. 907452, potency 842 mg/g) and of several metabolites (cf. fig. 1) were kindly supplied by Bayer AG, Wuppertal, FRG. Tetrabutyl ammonium phosphate (PIC A, low UV grade) was obtained from Waters GmbH., Eschborn, FRG. Unless otherwise stated reagents and solvents were of reagent grade purity supplied by E. Merck AG, Darmstadt, FRG. Redistilled water was used in all experiments.

Volunteers and patients

Volunteers participating in pharmacokinetic studies were instructed not to take any drugs or caffeine- or quinine-containing beverages 24 h before and during the study. Patients were on a regular hospital diet and usually received 4 to 6 drugs concomitantly which were recorded for possible analytical interferences. All volunteers gave written informed consent according to legal requirements in the FRG.

Blood was taken without additives and was allowed to clot at room temperature. It was subsequently centrifuged at 3000 *g* for 10 min. Serum and urine specimens for chromatography were stored at −80 °C for no more than 2 months before processing. The microbiological determination was performed on the day of sampling or on the day thereafter.

Preparation of the mobile phase

Solution of tetrabutyl ammonium phosphate: 5 mmol tetrabutyl ammonium phosphate (1 vial PIC A, Waters) were dissolved in approximately 800 ml water. The solution was adjusted to pH 2 with approximately 1.3 ml concentrated phosphoric acid and made up to 1 l.

Method A

125 ml acetonitrile were mixed with tetrabutyl ammonium phosphate solution and made up to a final volume of 1 l.

Method B

220 ml acetonitrile were mixed with tetrabutyl ammonium phosphate solution and made up to a final volume of 1 l.

The mobile phases were filtered before use and were recycled for no more than 3 days of continuous operation.

Preparation of standards

A stock solution of 10 mg/l ciprofloxacin was made by diluting 11.88 mg of reference material in water. The stock solution was stable at 4 °C for at least 1 week. Working standards (0.010 to 3.000 mg/l) were prepared daily by dilution of the stock standard with water. A standard solution of metabolite M3 was prepared by dissolving reference material in 0.5 ml 0.1 mol/l sodium hydroxide and further dilution with 5 mmol/l tetrabutyl ammonium phosphate solution. M1 was dissolved in 0.1 mol/l hydrochloric acid.

Processing of samples

Urine was diluted with aqueous tetrabutyl ammonium phosphate solution 1 : 40 to 1 : 10000 according to the expected concentration. Serum was deproteinised by mixing 0.3 ml serum with 0.6 ml acetonitrile. After centrifugation (2 min at 10000 *g*) 0.3 ml supernatant was diluted with 1.2 ml aqueous tetrabutyl ammonium phosphate solution. Standard solutions were treated likewise. To measure the extraction rate 0.3 ml drug-free serum was spiked with 0.1 ml standard solution (0.400 mg/l) and processed as described before. For control of precision, serum and urine pools of various concentrations were prepared from material obtained from volunteers.

High performance liquid chromatography (HPLC)

The chromatograph consisted of the following modules: a pump (flow rate 1 ml/min, pressure 15 MPa, model 2/1, Perkin Elmer, Überlingen, FRG); an automatic sampler (injection volume 20 µl (urine) or 20 µl or 50 µl (serum), model LC 420, Perkin Elmer); a precolumn (4 by 40 mm, Perisorb RP18, particle diameter 30—40 µm, E. Merck); a reversed phase column operated at room temperature 4 by 125 mm, Nucleosil 5C18, particle diameter 5 µm, Macherey & Nagel, Düren); a fluorescence detector (excitation 275 nm, emission cut-off 418 nm, time constant 4—6 s, model FS 970, Schoeffel GmbH, Karlsruhe); and an integrator (model 3390A, Hewlett Packard, Frankfurt). For method B a variable wavelength detector (lambda 280 nm, model LC 85, Perkin Elmer) was used. Concentrations were calculated from peak areas. Serum results were corrected for extraction rate. In sera from patients an extraction ratio of 0.95 was assumed.

Microbiological assay of ciprofloxacin

A microbiological assay which served as comparison was performed with a standard agar plate diffussion technique (12). The test species was *Klebsiella pneumoniae* ATCC 10031 for concentrations below 0.15 mg/l and *Bacillus subtilis* ATCC 6633

for concentrations above 0.15 mg/l. Samples and standards were assayed in triplicate. For qualitative detection of antimicrobial activity in chromatographic fractions a commercial test strip (Micur® BT, Boehringer Mannheim, FRG) was used.

Statistical calculations

Bivariate regression analysis was performed as previously described (13).

Results

Development of the method

Ciprofloxacin showed a typical UV absorption spectrum with a maximum at 275 nm. Upon excitation at 275 nm an intense blue fluorescence was observed with a maximum at 450 nm. For determination of the parent compound and the metabolite M1 (cf. fig. 1) detection by fluorescence was used in method A. The main metabolite M3, however, yielded a very low fluorescence and it was therefore quantitated by UV absorption in method B. The separation principle of both methods was reversed phase chromatography. Typical chromatograms from serum and urine show the separation of ciprofloxacin and the metabolites M1 and m2 from endogenous compounds by method A (fig. 2 and fig. 3). Addition of tetrabutyl ammonium phosphate to the mobile phase was essential to avoid tailing of the peaks. Retention times of fluorescent compounds encountered in monitoring ciprofloxacin therapy of patients are given in table 1.

Only tyrosine eluted close to ciprofloxacin. However, a high serum concentration of 100 µmol/l tyrosine produced a detector response equivalent to only 3 µg/l ciprofloxacin. For the elution of M3, the main metabolite in urine, the concentration of acetonitrile in the mobile phase had to be increased. Typical chromatograms obtained by method B using UV detection are shown in figure 4. Peaks of ciprofloxacin and M1 were qualitatively identified in 1 ml

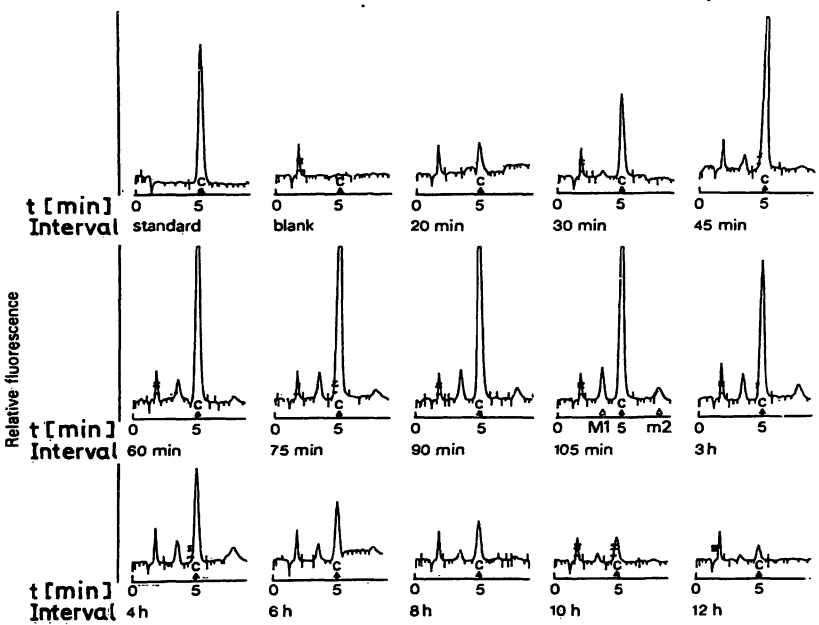

Fig. 2. Examples of chromatograms. Serum of a normal volunteer after a single oral dose of 750 mg ciprofloxacin. Separation of ciprofloxacin (C), M1 and m2 by method A.
t = retention time; the interval indicated below each chromatogram refers to sampling time after intake of the drug.

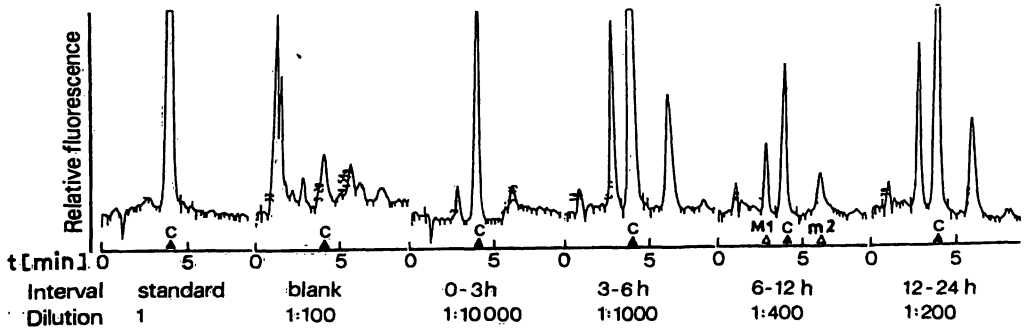

Fig. 3. Examples of chromatograms. Urine of a normal volunteer after a intravenous oral dose of 100 mg ciprofloxacin. Separation of ciprofloxacin (C), M1 and m2 by method A.
t = retention time; the interval indicated below each chromatogram refers to sampling period after intake of the drug; Dilution = sample dilution.

Tab. 1. Retention times of ciprofloxacin and various fluorescent compounds. Method A.

Compound	Retention time (min)	Relative retention time
Tryptophan	1.44	0.40
Metabolite M1	2.54	0.71
Ciprofloxacin	3.60	*1.00*
Tyrosin	3.87	1.08
Metabolite m2	5.72	1.59
Paracetamol	6.32	1.76
Doxycyclin	8.10	2.25
Metamizole	26.59	7.39
Salicylic acid	34.80	9.67

Retention times are relative to ciprofloxacin.

fractions collected after chromatography by UV spectroscopy and by demonstration of antibacterial activity, and also by addition of pure substances to the sample.

Linearity and sensitivity

Detection limits of ciprofloxacin by method A – estimated from the smallest detectable area by the integrator – were 10 µg/l in serum and 200 µg/l in urine at the most sensitive setting of the fluorimeter. Detector response measured by peak areas was linearly proportional to concentration over a range of 1 to 100. For very low concentrations the injection volume could be increased from 20 to 50 µl without distortion of the chromatogram. The detection limit of M3 by method B was 1 mg/l in urine.

Precision and accuray

Within-batch precision was measured in two serum pools, each in 10 replicates. Coefficients of variation were 0.8% (c = 1.45 mg/l) and 2.4% (c = 0.42 mg/l) for the complete protocol. For additional results see table 2. Between-batch precision was also measured in several serum and urine pools. Results are summarised in table 3. For serum concentrations of ciprofloxacin between 0.35 and 2.2 mg/l coefficients of variation varied from 4.8 to 9.3% and for urine samples coefficients of variation were between 2.4 and 7.2%. The average recovery of ciprofloxacin from 24 spiked drug-free sera was 94.5 ± 2.6% of the added concentration (c = 1.875 mg/l). In another two series of 12 and 9 sera, the mean recoveries were 97.2 ± 1.1% and 95.0 ± 1.8%. Recovery of ciprofloxacin from spiked urine was 99.6%.

Comparison of methods

In several hundred samples from healthy volunteers concentrations of ciprofloxacin were determined both by chromatography and a conventional microbiological assay. Results of bivariate regression analysis and of the sign test of paired differences are summarised in table 4. Examples of representative data sets are shown in figures 5 and 6. With the exception of two data sets obtained after a low dose (50 mg, urine) all bioassay results were significantly higher than the results obtained by chromatography.

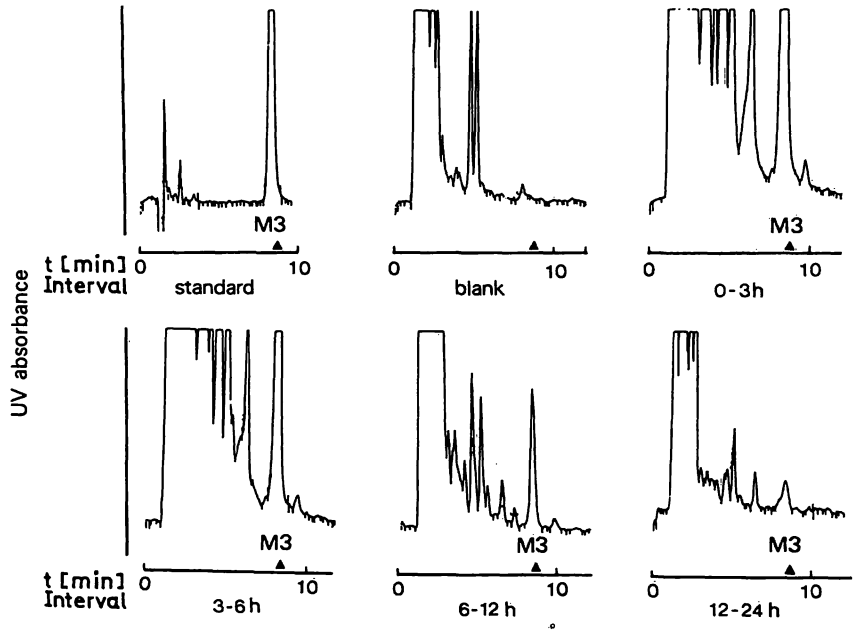

Fig. 4. Examples of chromatograms. Urine of a normal volunteer after a single oral dose of 250 mg ciprofloxacin. Determination of metabolite M3 by method B.
t = retention time; the interval indicated below each chromatogram refers to sampling period after intake of the drug.

Tab. 2. Within-batch precision of method A.

Material	Component	N	$\bar{x}$ (mg/l)	CV (%)
Serum	ciprofloxacin	10	1.45	0.8
Serum	ciprofloxacin	10	0.42	2.4
Serum	metabolite M1	10	0.010	6.9
Serum	metabolite m2	11	(0.047)	25.8
Urine	ciprofloxacin	10	209.0	2.1
Urine	ciprofloxacin	10	29.4	1.7
Urine	metabolite M1	10	6.2	5.5
Urine	metabolite M1	10	1.4	1.9
Urine	metabolite m2	10	(79.9)	2.1
Urine	metabolite m2	10	(18.2)	3.2

Concentrations of m2 are only given in fluorescence equivalents relative to ciprofloxacin (figures in brackets).

Tab. 3. Between-batch precision of method A.

Material	Component	N	$\bar{x}$ (mg/l)	CV (%)
Serum	ciprofloxacin	10	1.79	4.8
Serum	ciprofloxacin	10	0.40	6.0
Serum	ciprofloxacin	13	1.84	7.4
Serum	ciprofloxacin	12	0.41	8.3
Serum	ciprofloxacin	5	2.24	9.3
Serum	ciprofloxacin	11	0.57	7.6
Serum	ciprofloxacin	14	0.33	6.0
Urine	ciprofloxacin	19	118.8	2.4
Urine	ciprofloxacin	15	2.26	7.2
Urine	metabolite M1	18	16.4	12.0
Urine	metabolite M1	16	1.04	8.4
Urine	metabolite m2	15	(17.2)	12.9
Urine	metabolite m2	15	(0.92)	16.6

Concentrations of m2 are only given in fluorescence equivalents relative to ciprofloxacin (figures in brackets).

Tab. 4. Comparison of methods. HPLC (x) versus bioassay (y). Statistical evaluation.

Dose	N	HPLC	Bioassay	Sign test	Bivariate regression analysis	
mg		$\bar{x}$ (mg/l)	$\bar{x}$ (mg/l)	p	a (mg/l)	b
Urine						
50 p. o.	48	18	22	> 0.20	− 1.4	1.290
50 i. v.	48	35	38	> 0.20	+ 1.3	1.042
100 p. o.	48	49	63	< 0.01	− 3.3	1.359
100 i. v.	46	80	98	< 0.01	+ 2.2	1.196
250 p. o.	40	193	252	< 0.01	+ 9.2	1.262
250 p. o.	40	112	175	< 0.01	+ 0.7	1.556
750 p. o.	48	297	375	< 0.001	− 0.6	1.264
Serum						
50 i. v.	202	0.232	0.255	< 0.05	− 0.002	1.107
100 p. o.	178	0.212	0.239	= 0.05	− 0.019	1.213
100 i. v.	201	0.537	0.598	< 0.01	− 0.010	1.134
250 p. o.	160	0.532	0.660	< 0.01	− 0.036	1.309
750 p. o.	166	1.197	1.543	< 0.001	+ 0.023	1.271

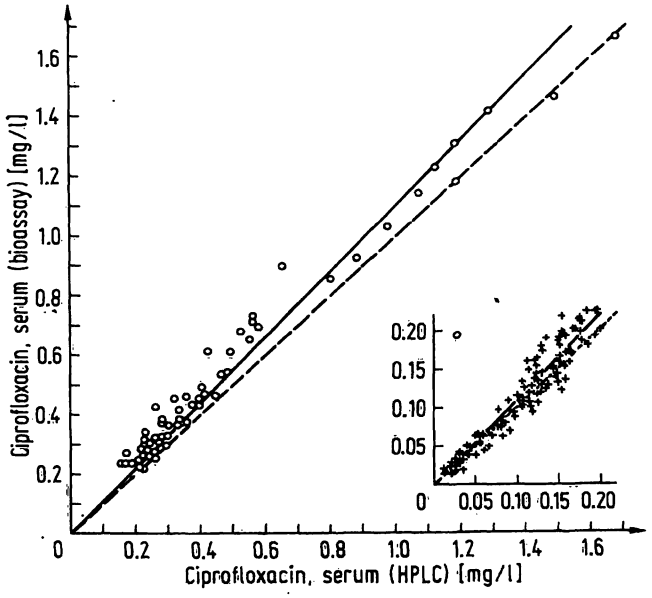

Fig. 5. Comparison of methods. Ciprofloxacin in serum after a single intravenous dose of 50 mg. Bivariate regression analysis: y (bioassay) = 1.107x (HPLC) + 0.002 mg/l.

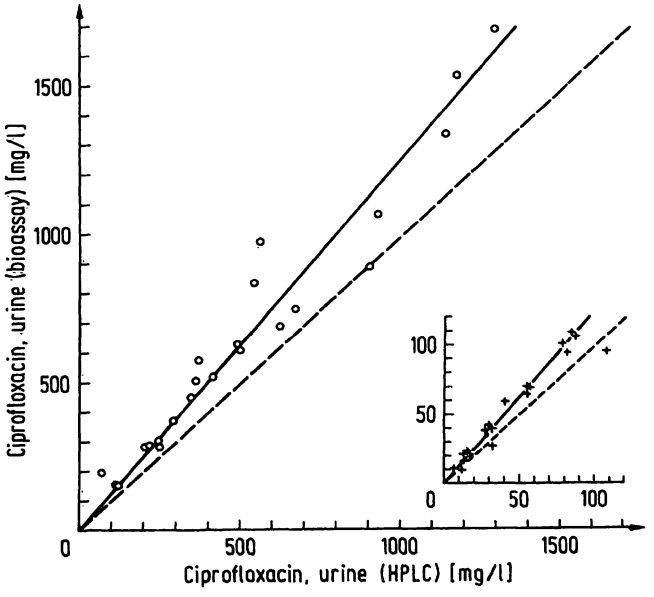

Fig. 6. Comparison of methods. Ciprofloxacin in urine after a single oral dose of 750 mg. Bivariate regression analysis: y (bioassay) = 1.264x (HPLC) − 0.6 mg/l.

Interferences

Up to now, ciprofloxacin has been determined in more than 2000 samples, serum and urine, from healthy volunteers without any interference. In another study on 60 patients there was only one unexplained analytical interference in a patient with terminal renal insufficiency, who had received several other drugs simultaneously.

Discussion

Ciprofloxacin is a very potent antimicrobial agent as compared with other groups of antibiotics (1–8). E. g., MIC90 values of 0.015 to 2 mg/l were reported for several *Gram*-negative species (2). Thus, a fairly sensitive method was required for its determination in human body fluids. Furthermore it had to be taken into account that ciprofloxacin is partially metabolised by the human organism (9, 10). Some of its metabolites still showed activity in the microbiological assay used. Metabolites can also interfere in chromatographic methods. The chemical structures of three metabolites have so far been identified (cf. fig. 1) (14). Another metabolite is at present under investigation by co-workers of the manufacturer. This paper shows an additional possible metabolite called m2 the structure of which is unknown at present (cf. figs. 2 and 3). Metabolite M4 could not be detected in urine. According to l. c. (14) its renal excretion is less than 1% of the dose.

Method A uses fluorescence detection for high sensitivity and better specificity. It differs from a method first described by *Gau* (15) in the method of deproteinisation, the stationary phase and completeness of separation. During the preparation of the manuscript similar chromatographic methods for ciprofloxacin have been published (16–18), none of which deals with the quantitation of the metabolites. The present method A separated ciprofloxacin from all known metabolites (cf. figs. 2 and 3). It was sufficiently sensitive to measure serum concentrations up to 24 h after administration of a single oral dose of 250 mg. Precision and recovery were satisfactory. Since the quantum yield of the metabolites differs considerably from that of the parent compound, calibration of the fluorescence detector with pure reference substances is essential. The poor precision obtained with metabolite m2 in serum (tab. 2) was probably due to the rather small signal relative to the parent compound to which the fluorometric detector was adjusted (cf. fig. 2).

Method B was developed for the main metabolite M3 (oxo-ciprofloxacin), which yielded low fluorescence and was less polar (cf. fig. 4).

Results of the microbiological assay performed with split samples were generally somewhat higher than results obtained by chromatography (tab. 4). The differences were more pronounced in urine than in serum. This is likely to be due to the presence of metabolites with antimicrobial activity, the concentration of which is usually higher in urine than in serum. In one trial a single oral dose of 250 mg ciprofloxacin was given to 10 volunteers. The mean renal elimination within 24 h was ciprofloxacin 33.6 ± 8.0% of the dose, metabolite M1 1.3 ± 0.5% and metabolite M3 5.5 ± 8.0% (11). Metabolite M3 had an activity of 74% relative to ciprofloxacin. Similar differences between microbiological and chromatographic results were reported for urine (16) and for bile (18). For this reason liquid chromatography appears the more specific method for ciprofloxacin and is recommended both for pharmacokinetic studies and therapeutic drug monitoring. For the latter application some caution is warranted in patients on concomitant treatment with drugs that also fluoresce. Since clinical experience with ciprofloxacin is presently still limited, indications for routine therapeutic drug monitoring are not yet defined. Possible indications might be high dose therapy in severe infections and/or reduced elimination capacity in renal or hepatic disease.

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Prof. Dr. K. Borner
Institut für Klinische Chemie
und Klinische Biochemie
Hindenburgdamm 30
D-1000 Berlin 45